

### **REMARKS / ARGUMENTS**

The Final Office Action mailed February 22, 2006, was received and reviewed. Claims 1 through 34 are pending in the Application, and claims 1 through 15 are withdrawn from consideration as being directed to a non-elected invention. Claims 16 through 34 were rejected. Claims 30 and 34 are being amended as shown above.

A telephonic interview was conducted over this case on April 4<sup>th</sup>, 2006, and a statement of the substance of this interview is provided below.

Applicants hereby respectfully request reconsideration of the amended Application, and offer the following remarks and arguments for consideration by the Examiner.

#### **STATEMENT OF THE SUBSTANCE OF THE TELEPHONIC INTERVIEW OF APRIL 4, 2006**

A telephonic interview concerning this Application was held on April 4, 2006. In attendance were Examiner Louis V. Wollenberger and Primary Examiner Doug Schultz, representing the United States Patent and Trademark Office; and Herbert L. Ley III and Jay Z. Zhang, representing the Applicants.

Under discussion were the rejections presented in the Final Office Action, mailed February 22, 2006. Specifically, rejections under 35 USC § 112, 2<sup>nd</sup> paragraph – Definiteness; 35 USC § 112, 1<sup>st</sup> paragraph – Written Description; and 35 USC § 102(b) – Novelty, in view of Cox *et al.*, *Genes & Development* 12:3715-3727 (1998), were discussed. Also discussed was the finality of the Final Office Action.

#### **FINALITY OF THE LAST OFFICE ACTION**

During the telephonic interview mentioned above, Applicants formally requested reconsideration of the finality of the Final Office Action. Applicants' grounds for requesting reconsideration were based on the fact that the Final Office Action issued a 35 USC § 102(b) anticipation rejection citing, for the first time, prior art by Cox *et al.*, (*Genes & Development* 12:3715-3727 (1998)), which, in the opinion of the Applicants, could have been cited in the previous Office Action, was not necessitated by an amendment, and does not anticipate the claimed invention (as explained below).

Applicants' arguments were deemed persuasive, and Examiners Wollenberger and Schulz agreed that the Final Office Action mailed February 22, 2006, was prematurely made final. Consequently, the Examiners indicated that another Office Action would be prepared and mailed and that this new Office Action would withdraw the finality of the preceding Office Action and reopen the prosecution of the Application. Further confirmation of this decision was received by Applicants in the form of a telephone call from Primary Examiner Sean McGarry approximately two days after the telephonic conference took place.

#### **CLAIM AMENDMENTS**

Claims 30 and 34 have been amended, as shown above, by the deletion of the term "FLAG®." These amendments were made to obviate the rejection under 35 USC § 112, 2<sup>nd</sup> paragraph – Definiteness.

The amendments should be entered into the record because they neither add new matter to the Application, nor raise new issues that would require further search, and they place the claims in condition for allowance, or, alternatively, in better condition for appeal.

#### **THE REJECTIONS**

##### **35 USC § 112, 2<sup>nd</sup> paragraph – Definiteness:**

Claims 30 and 34 stand rejected under 35 USC § 112, 2<sup>nd</sup> paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The amendment of claims 30 and 34 removes the offending trademark/trade name "FLAG®," thereby obviating the rejection. Applicants respectfully note, however, that the term "FLAG," as used in the context of epitope tags, has had clear meaning in the art since at least 1994, when Brizzard and colleagues, of the Eastman Kodak Company, taught, in the abstract of a printed publication, that "[t]he FLAG epitope is an eight amino acid peptide (AsnTyrLysAspAspAspAspLys) that is useful for immunoaffinity purification of fusion proteins." (See Brizzard *et al. Biotechniques* 16(4):730-5, (1994)), abstract, provided herewith as *Exhibit A.*)

**35 USC § 112, 1<sup>st</sup> paragraph – Written Description:**

Claims 16-34 stand rejected under 35 U.S.C. § 112, 1<sup>st</sup> paragraph for being based upon a specification that allegedly provides insufficient written description.

As a first matter, compliance with the “written description requirement” is a question of fact. For this reason, the MPEP § 2163.04 notes: “The examiner has the initial burden of presenting **by a preponderance of evidence** why a person skilled in the art would not recognize in an applicant’s disclosure a description of the invention defined by the claims. *Wertheim*, 541 F.2d at 263, 191 USPQ at 97.” (MPEP, 8<sup>th</sup> Ed. Rev. 3, August 2005, pp. 2100-186-2100-187; emphasis added). MPEP § 2163.04 further instructs:

In rejecting a claim, the examiner must set forth **express findings of fact** which support the lack of written description conclusion .... **A general allegation of "unpredictability in the art" is not a sufficient reason to support a rejection for lack of adequate written description.**

(MPEP, 8<sup>th</sup> Ed. Rev. 3, August 2005, pp. 2100-187; emphasis added).

Applicants respectfully assert that, in the present case, the Final Office Action fails to provide any **evidence or express findings of fact** as to why a person skilled in the art would not recognize in an applicant’s disclosure a description of the invention defined by the claims. Further, Applicants assert that the arguments in the Final Office Action comprise little more than allegations of unpredictability in the art.

As a second matter, the United States Court of Appeals for the Federal Circuit has recently made clear the following three points in *Capon v. Eshhar* (418 F.3d 1349, Fed. Cir. 2005). First, “[t]he descriptive text needed to meet [the written description requirement] varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence.” *Id* at 1357. Second, “[t]he written description requirement may be satisfied “if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure.” *Id* at 1357. Third, “[i]n the patent context, the written description **must** be applied in the context of the particular invention and the state of the knowledge.” *Id* at 1358.

Additionally, the United States Patent and Trademark Office (PTO) has issued guidelines for the examination of patent applications under the 35 USC § 112, first paragraph, written description requirement. These guidelines state that the written description requirement of 35 USC § 112, first paragraph, can be met by

show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics ... i.e., complete or partial structure, other physical and/or chemical properties, **functional characteristics when coupled with a known or disclosed correlation between function and structure**, or some combination of such characteristics.

Guidelines for Examination of Patent Applications under 35 USC § 112, first paragraph, “Written Description” Requirement, 66 Fed. Reg. 1099, 1106 (Jan. 5, 2001) (emphasis added).

Applicants respectfully note that the nexus of the instant invention was the realization that the expression of any number of recombinant proteins from chimeric RNA transcripts could be disrupted by RNA interference induced by the action of a single, pre-selected, “universal interfering RNA” (i.e., an siRNA or shRNA), if that universal interfering RNA targeted a commonly shared sequence incorporated into the plurality of chimeric RNA transcripts. Applicants further note that the “fundamental elements” of the invention, include (a) the shared, common target sequence (a “universal target sequence” that can be located essentially anywhere in the recombinant transcript), and (b) the corresponding universal interfering RNA that acts by targeting the universal target sequence and inducing RNA interference – and such elements were already available to the skilled artisan at the time the invention was made.

Furthermore, Applicants respectfully remind the Examiner that the pending claims are drawn towards kits for practicing a novel method – and are NOT drawn towards novel compositions of matter such as novel genes, novel segments of DNA, or even novel siRNAs or shRNAs. Consequently, holding the instant specification to the standard of written description required for novel genes, novel segments of DNA, or even novel siRNAs or shRNAs would be inappropriate. As the specification makes amply clear, and as the pending claims suggest, known nucleic acids can be used in the claimed kits and methods of the invention, both in the context of universal target RNAs and the

interfering siRNAs and shRNAs that target them. In other words, the concept of “RNA interference using a universal target” does not require the development of new nucleic acid components with novel activities, instead, it can encompass the use of new combinations of previously known components. (See below for an example.)

**State of the Art:**

Applicants respectfully assert that the “written description rejection,” as presented in the Final Office Action, essentially ignores the state of knowledge in the relevant arts at the time the instant Application was filed. Additionally, the Final Office Action fails to recognize that the written description requirement may be satisfied “if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure.” *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1332 (Fed. Cir. 2003); as recited in *Capon v. Eshhar*, 418 F.3d 1349 (Fed. Cir. August 12, 2005).

Applicants respectfully note that at the time the instant Application was filed, skilled artisans were well aware that RNA interference was induced by siRNAs and shRNAs with particular, known structures. Further, the instant Application teaches in detail (in Section 7, pages 39 - 41) the general structures of siRNAs and shRNAs required to induce RNA interference, and the methods by which they can be created and introduced into cells, tissues or organisms. The specification also provides references to critical and informative publications providing additional teachings as well as clear documentation as to what was within the purview of the skilled artisan at the time the instant Application was filed.

In addition to these teachings, as indicated above, the prior art provides all the necessary elements to create embodiments of the instant invention, and further provides sufficient proof of concept to demonstrate that the instant invention is adequately described. Specifically, the prior art contains descriptions of examples of chimeric RNA transcripts comprising a “subject RNA” operably linked to a “target RNA,” which do not naturally occur in nature, and which can be targeted for degradation by RNA interference with an siRNA or shRNA that corresponds in sequence to at least a portion of the operably linked target RNA, and not the subject RNA. Thus, in essence, the prior art puts

skilled artisans in possession of all of the necessary elements of the invention, however, the prior art fails to teach that the same siRNA or shRNA can be used to target a plurality of chimeric RNA transcripts, each bearing a different subject RNA operably linked to a shared, “universal,” target RNA.

As evidence in support of this assertion – that the necessary elements required to create an embodiment of the instant invention were available in the art prior to the filing of the instant Application – Applicants first note that the instant Application states “For example, the [universal target RNA] can encode enhanced green fluorescent protein, or any other variety of fluorescent protein.” (Specification, page 38, lines 10-11.) Applicants next note that in an article published on August 14, 2001, Caplen *et al.*, taught that enhanced green fluorescent protein (EGFP) recombinantly-expressed in mouse embryonic fibroblasts can be effectively knocked down using chemically-synthesized siRNAs corresponding to the target region comprising nucleotides 122-141 of the EGFP coding sequence (GCAAGCUGACCCUGAAGUUC) (Caplen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 98:9742-9747 (2001); provided herewith as *Exhibit B*). (This same target sequence was also effectively targeted in HeLa cells recombinantly-expressing EGFP by Donzé & Picard, using enzymatically-synthesized siRNA of the same sequence, according their May 15, 2002 publication (Donzé & Picard. *Nucleic Acids Res.* 30:e46 (2002); provided herewith as *Exhibit C*).

As further evidence that these “prior art elements” can be used to practice the methods of the instant invention, Applicants note these same prior art elements reappear in an article describing a “new and universal transgene silencing method based on RNA interference,” which was published 9 months after the filing of the instant Application. (See Mangeot *et al.*, *Nucleic Acids Res.* 32:e102 (2004); which was provided to the Office in the IDS filed March 3, 2004). In demonstrating the effectiveness of this method for silencing transgene expression, Mangeot *et al.*, employ an shRNA that targets the same 20 nucleotides of the EGFP coding sequence targeted by Caplen *et al.* and Donzé & Picard. However, in this embodiment of a “universal transgene silencing method,” the EGFP target sequence is not present within the context of a transcript that only encodes full-length EGFP (as it was in the studies of Caplen *et al.* and Donzé & Picard), but rather, it is contained within the context of either a bicistronic transcript encoding human

thioredoxin and full-length EGFP, or, alternatively, it is embedded, as part of a 25 nucleotide fragment of the EGFP coding sequence, within the 3'-untranslated region of a chimeric RNA transcript encoding human thioredoxin.

In view of these facts, Applicants assert that it is amply clear that the knowledge of the art provides a disclosed function [induction of RNA interference], which has been sufficiently correlated to a particular, known structure [specific target RNAs, and the siRNAs and shRNAs act on that target to induce RNA interference]. Hence, the teachings of the art satisfy the written description requirement, with respect to the elements required to practice the claimed invention.

**Improper *prima facie* case for written description rejection:**

As noted above, MPEP § 2163.04 states that in establishing a *prima facie* case for insufficient written description: "The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. *In re Wertheim*, 541 F.2d at 263, 191 USPQ at 97." MPEP § 2163.04 also makes clear that "A general allegation of "unpredictability in the art" is not a sufficient reason to support a rejection for lack of adequate written description."

Applicants respectfully assert that the Final Office Action presents no factual evidence indicating why a person skilled in the art would not recognize in the Applicants' disclosure, a description of the invention defined by the claims. Further, Applicants assert that the arguments in the Final Office Action amount to no more than a general allegation of unpredictability in the art.

While the Final Office Action (on pages 7 and 8) presents arguments on the inherent unpredictability in art as it relates to siRNA structure and function, these arguments, respectfully, are not relevant to the claimed invention, and actually lend support to the claims, at least in terms of utility. Specifically, the Final Office Action cites two passages from the instant Application in which Applicants have argued that (a) the *a priori* selection of target sequences within gene transcripts to target with siRNAs and shRNAs is problematic, and (b) the *a priori* design of siRNAs and shRNAs to effectively target specific sequences within target gene transcripts is problematic.

Applicants note that the instant invention is designed to specifically address and solve these problems by eliminating the need to select target sequences in the desired chimeric RNA transcripts and design siRNAs and shRNAs directed to those target sequences, *a priori*. Instead, the claimed invention encompasses (1) using siRNAs and shRNAs which are already known to effectively target specific target sequences, and (2) making a plurality of chimeric RNA transcripts that will be susceptible to RNA interference induced by such siRNAs and shRNAs, by simply engineering the sequence they are known to effectively target into the chimeric RNA transcripts.

As Applicants have noted above, the prior art provides specific examples of such siRNAs and shRNAs, and their corresponding target RNAs, that can be used in the kits and methods of the instant invention. (See *Exhibits B and C* and Mangeot *et al.*, *Nucleic Acids Res.* 32:e102 (2004); provided to the Office in the IDS filed March 3, 2004.)

In summary, it is submitted that the descriptions provided in the specification constitute sufficiently detailed, relevant identifying characteristics of the claimed subject matter consistent with *Enzo*, and the USPTO's own "Written Description Guidelines." It is also submitted that the instant Office Action has failed to establish why one of ordinary skill in the art, provided with the descriptions of the specification, combined with the teachings of the prior art, would be unable to recognize invention defined by the claims. Finally, as stated by the Federal Circuit in *Capon v. Eshhar*:

"The "written description" requirement must be applied in the context of the particular invention and the state of the knowledge. ... When the prior art includes nucleotide information, precedent does not set a per se rule that the information must be determined afresh. ... As each field evolves, the balance evolves between what is known and what is added by each inventive contribution. ... It must be borne in mind that, while it is necessary that an applicant for a patent give to the public a complete and adequate disclosure in return for the patent grant, the certainty required of the disclosure is not greater than that which is reasonable, having due regard to the subject matter involved."

*Capon v. Eshhar*, 418 F.3d 1349 (Fed. Cir. August 12, 2005); at 1358 & 1360.

Accordingly, Applicants assert that claims 16-34 are based upon a specification that provides adequate written description of the claimed invention, and request that written description rejection under 35 USC § 112, first paragraph, be withdrawn.

**35 USC § 102(b) – Novelty in view of Cox *et al.***

Claims 18-20 and 31 stand rejected under 35 USC § 102(b) as allegedly being anticipated by Cox *et al.*, *Genes & Development* 12:3715-3727 (1998) (hereinafter referred to as Cox), which discloses the use of a single “anti-*prg-1* RNA” to interfere with the function of the two highly similar *prg-1* and *prg-2* genes in the nematode, *C. elegans*, by inducing RNA interference.

Applicants respectfully assert that Cox does not anticipate the instant invention because of two critical differences between that which is disclosed in Cox, and the claimed invention: First, the *prg-1* and *prg-2* gene transcripts of Cox are not “chimeric transcripts” as defined by the instant specification, but instead are naturally-occurring native transcripts from highly similar paralogous genes. Second, the single “anti-*prg-1* RNA” used by Cox to interfere with the function of the *prg-1* and *prg-2* genes by inducing the RNAi mediated degradation of the *prg-1* and *prg-2* transcripts, is neither an siRNA nor an shRNA, but is, instead, a long double-stranded RNA. These two critical differences will now be discussed in detail.

With regard to the first difference, Cox does not anticipate the claimed invention because the two transcripts being targeted by the same injected “anti-*prg-1* RNA” are the naturally-occurring endogenous transcripts transcribed from the paralogous *prg-1* and *prg-2* genes; which do not constitute **chimeric RNA transcripts** according to the definition provided on page 16, line 28-30, of the Application. Specifically, that definition states: “As used herein, the term “chimeric RNA transcript” means an RNA transcript comprising a subject RNA operably linked to a universal target RNA to create a single RNA that does not naturally occur in nature.” Since the *prg-1* and *prg-2* gene transcripts targeted in the RNAi experiments of Cox are naturally occurring RNA molecules, they are specifically excluded by the definition provided and thus do not fall within the scope of the claims.

Applicants respectfully remind the Examiner that MPEP § 2173.05(a) provides:

“During patent examination, the pending claims must be given the broadest reasonable interpretation consistent with the specification. ...

**When the specification states the meaning that a term in the claim is intended to have, the claim is examined using that meaning**, in order to achieve a complete exploration of the applicant's invention and its relation

to the prior art. *In re Zletz*, 893 F.2d 319, 13 USPQ2d 1320 (Fed. Cir. 1989).”

MPEP 8<sup>th</sup> Edition, Rev. 3, August 2005, p. 2100-215 – 2100-216.

Additionally, Applicants assert that regardless of the definition of chimeric transcript provided in the specification, the art accepted definition of “chimeric gene” requires that it be a combination of segments of DNA, combined in a way that does not occur in nature. Specific evidence in support of this assertion can be found in the Federal Circuit’s *Capon v. Eshhar* decision, which states: “A chimeric gene is an artificial gene that combines segments of DNA in a way that does not occur in nature.” *Capon v. Eshhar*, 418 F.3d 1349 (Fed. Cir. 2005), at 1351; emphasis added.

With regard to the second difference mentioned above, Cox does not anticipate the kits of claims 18-20 and 31, because the “anti-*prg-1* RNA” used to disrupt the function of the *prg-1* and *prg-2* genes is not an siRNA or an shRNA according to the definitions provided in the specification and the commonly accepted meaning of the terms in the art. Instead, the “anti-*prg-1* RNA” used in these experiments is presumably a dsRNA of at least 2,472 bp (the equivalent length of the *prg-1* open reading frame), comprised of “an equimolar mixture of uncapped sense and antisense RNA” transcribed from the convergent promoters of the pBluescript KS(+) transcription vector. (See Cox, “RNAi experiments” subsection of the “Materials and methods” section; p. 3725, righthand column.)

In view of these critical differences between the claimed invention and what is disclosed in Cox, Applicants contend that teachings of Cox do not anticipate the claimed invention. Therefore, the rejection of Claims 18-20 and 31 under 35 USC § 102(b) in view of Cox, should be withdrawn.

### CONCLUSIONS

Claims 16 through 34 are believed to be in condition for allowance, and an early notice thereof is respectfully solicited. Should the Examiner determine that additional issues remain which might be resolved by a telephone conference, he is respectfully invited to contact the undersigned via his direct office line (801-883-3463). Additionally, should either claim 16 or 18, or both, be found to be allowable, Applicant respectfully requests rejoinder and examination of withdrawn process (method) claims (i.e., claims 1-15), in accordance with the provisions of MPEP § 821.04.

It is believed that no other extension of time, nor additional fee, is due with this response. If this is incorrect, an extension of time as deemed necessary is hereby requested, and the Commissioner is hereby authorized to charge any appropriate fees or deficiency or credit any over payment to Deposit Account no. 50-1627.

Respectfully submitted,



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May 19, 2006

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1: Biotechniques. 1994 Apr;16(4):730-5.

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## Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution.

Brizzard BL, Chubet RG, Vizard DL.

Eastman Kodak Company, New Haven, CT.

The FLAG epitope is an eight amino acid peptide (AspTyrLysAspAspAspLys) that is useful for immunoaffinity purification of fusion proteins. A monoclonal antibody (anti-FLAG M1) that binds the FLAG epitope in a calcium-dependent manner and requires an N-terminal FLAG sequence has been described previously. We describe the use of a second anti-FLAG monoclonal antibody (anti-FLAG M2) in immunoaffinity purification of N-terminal Met-FLAG and C-terminal FLAG fusion to bacterial alkaline phosphatase. Although binding of an anti-FLAG M2 monoclonal antibody to the FLAG epitope is not calcium-dependent, bound fusion proteins can be eluted by competition with FLAG peptide.

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May 9 2006 14:13:00

# Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems

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Edited by Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD, and approved June 21, 2001 (received for review May 18, 2001)

Short interfering RNAs (siRNAs) are double-stranded RNAs of ~21–25 nucleotides that have been shown to function as key intermediaries in triggering sequence-specific RNA degradation during posttranscriptional gene silencing in plants and RNA interference in invertebrates. siRNAs have a characteristic structure, with 5'-phosphate/3'-hydroxyl ends and a 2-base 3' overhang on each strand of the duplex. In this study, we present data that synthetic siRNAs can induce gene-specific inhibition of expression in *Caenorhabditis elegans* and in cell lines from humans and mice. In each case, the interference by siRNAs was superior to the inhibition of gene expression mediated by single-stranded antisense oligonucleotides. The siRNAs seem to avoid the well documented nonspecific effects triggered by longer double-stranded RNAs in mammalian cells. These observations may open a path toward the use of siRNAs as a reverse genetic and therapeutic tool in mammalian cells.

**M**echanisms that silence unwanted gene expression are critical for normal cellular function. Characterized gene silencing mechanisms include a variety of transcriptional and posttranscriptional surveillance processes (1–3). Double-stranded RNA (dsRNA) has been shown to trigger one of these posttranscriptional surveillance processes, in which gene silencing involves the degradation of single-stranded RNA (ssRNA) targets complementary to the dsRNA trigger (4). RNA interference (RNAi) effects triggered by dsRNA have been demonstrated in a number of organisms including plants, protozoa, nematodes, and insects (5). RNAi may play a role in the silencing of mobile elements in *Caenorhabditis elegans* and *Drosophila* (6–9). Similar posttranscriptional gene silencing (PTGS) effects have been implicated as an anti-viral response in plants. PTGS/RNAi seems to be a multistep pathway requiring the processing of the trigger, a facilitated interaction with, and degradation of, the target mRNA. In some cases, these processes may also involve physical amplification of the trigger RNA and long-term maintenance of gene silencing (10, 11).

A key finding from recent work has shown the generation of small (~21–25 nucleotides) dsRNAs from the input dsRNA during PTGS and RNAi (12–16). These small dsRNAs have been detected in plants, *Drosophila*, and *C. elegans* and have been suggested to serve as guide RNAs for target recognition. In *Drosophila* extracts subjected to RNAi, these small dsRNAs [called short interfering (siRNAs)] resemble breakdown products of an RNase III-like digestion (17). In particular, each strand of the siRNAs carry 5' phosphate and 3' hydroxyl termini and 2- or 3-nt 3' overhangs. siRNAs of 21–22 nucleotides can induce specific degradation when added to *Drosophila* cell extracts (17). Further, a *Drosophila* dsRNA-specific RNase has been identified that can degrade large dsRNA (200 and 500 bp) to small dsRNAs of ~22 nucleotides. RNAi-triggered inhibition of this ribonuclease significantly reduces the effectiveness of RNAi in *Drosophila* S2 cells (18).

As yet, clear evidence for the generality of an RNAi-like mechanism in vertebrate cells is lacking. Several studies have reported evidence for dsRNA-triggered silencing in particular certain vertebrate systems, early embryos of mice, zebrafish, and *Xenopus*, as well as Chinese hamster ovary cells (19–25). At the same time, numerous reports have described failures to observe gene-specific RNAi effects in different vertebrate systems, demonstrating instead nonspecific effects of dsRNA on gene expression (26–29). These nonspecific effects have not been surprising as there is an extensive literature describing a variety of nonspecific responses induced by dsRNAs in mammalian cells. A major component of the mammalian nonspecific response to dsRNA is mediated by the dsRNA-dependent protein kinase, PKR, which phosphorylates and inactivates the translation factor eIF2 $\alpha$ , leading to a generalized suppression of protein synthesis and cell death via both nonapoptotic and apoptotic pathways (30). PKR may be one of several kinases in mammalian cells that can mediate this response (31). A second dsRNA-response pathway involving the dsRNA-induced synthesis of 2'-5' polyadenylic acid and a consequent activation of a sequence-nonspecific RNase (RNaseL) has also been demonstrated (32). These nonspecific responses to dsRNA, however, do not necessarily preclude the presence of an RNAi-like mechanism in mammalian cells. The activation of PKR by dsRNA has been shown to be length-dependent; dsRNAs of less than 30 nucleotides are unable to activate PKR, and full activation requires ~80 nucleotides (33, 34). Given the observations that (i) 21–25-nt dsRNAs with a characteristic structure can mediate RNAi in cell extracts and that (ii) dsRNAs of less than 30 bp do not activate PKR, we set out to determine whether short dsRNAs with an RNase III cleavage structure could trigger a gene-specific RNAi response in model invertebrates and mammalian cells.

## Methods

**Nucleic Acids.** Single-stranded, gene-specific sense and antisense RNA oligomers were synthesized by using 2'-O-(tri-isopropyl) silyloxymethyl chemistry by Xeragon AG (Zurich, Switzerland). We have previously shown RNAs produced by this methodology are highly pure and efficiently form RNA duplexes (16, 27). For

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; siRNA, short interfering RNA; RNAi, RNA interference; PTGS, posttranscriptional gene silencing; GFP, green fluorescence protein; CAT, chloramphenicol acetyl transferase; PKR, dsRNA-dependent protein kinase; neo, neomycin phosphotransferase; MEF, mouse embryonic fibroblast; FACS, fluorescence-activated cell sorter.

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studies conducted in *C. elegans*, RNA oligomers were annealed and injected into adults at a concentration of 5 mg/ml as described (16). For experiments conducted using mammalian cells, dsRNA molecules were generated by mixing sense and antisense ssRNA oligomers (100  $\mu$ g each) in 10 mM Tris-Cl (pH 7.0), 20 mM NaCl (total volume 300  $\mu$ l), heating to 95°C, and cooling slowly (18 h) to room temperature. The dsRNAs were ethanol-precipitated and resuspended in water at  $\approx$ 0.5 mg/ml. The integrity and the dsRNA character of the annealed RNAs were confirmed by gel electrophoresis. The sequences of the RNA oligonucleotides used are shown in Table 2, which is published as supplemental data on the PNAS web site, www.pnas.org; the *cat* 22 and 23 ssRNA oligomers were HPLC-purified. Plasmid pEGFP-N3 (CLONTECH) expresses a mammalian-enhanced version of green fluorescent protein (GFP) and neomycin phosphotransferase (neo). Plasmid pcDNA3.CAT (Invitrogen) expresses chloramphenicol acetyl transferase (CAT) and neo.

**Cell Culture and Nucleic Acid Transfections.** All mammalian cells were grown in DMEM (Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) FBS (Gemini Biological Products, Calabasas, CA). Primary mouse embryonic fibroblasts (MEFs) from wild-type 1129 mouse embryos (a gift of J. Bell, Univ. of Ottawa, Ontario, Canada; ref. 31) were expanded to generate a more homogenous cell line and were used at passages 20–50 (35). 293 is a human embryonic kidney cell line (36); HeLa is a human epithelial cell line derived from a cervical adenocarcinoma [American Type Culture Collection (ATCC) no. CCL-2]. Plasmid/RNA cotransfection of mammalian cells was mediated by using the cationic lipid Lipofectamine (GIBCO) and the propriety plus reagent (Life Technologies). Cells were seeded  $\approx$ 18 h before transfection and were transfected at  $\approx$ 70–80% confluency. Plasmid DNA was complexed with the plus reagent (4–6  $\mu$ l/2  $\mu$ g DNA) in DMEM for  $\approx$ 15 min. RNAs were added 5–10 min into the plasmid/plus reagent incubation. Lipofectamine diluted in DMEM was added to the plasmid/plus reagent/RNA mixture, and complexation was continued for an additional 15 min. The amount of Lipofectamine added (8–15  $\mu$ g) was based on the total weight of nucleic acid (DNA and RNA) used and a weight to weight ratio of nucleic acid to lipid of 1:4. The amount of RNA used was adjusted to account for the variations in the sizes of RNA. For small RNAs (21–27 nucleotides), 70 pmols of ssRNA and dsRNA was used, corresponding to  $\approx$ 0.5  $\mu$ g of a 22-nt ssRNA and 1  $\mu$ g of 22-nt dsRNA. For the larger RNAs (78–81 nucleotides),  $\approx$ 30 pmols of RNA was used (0.85  $\mu$ g of ssRNA and 1.7  $\mu$ g of dsRNA). Three hours after initiation of transfection, DMEM supplemented with 20% (vol/vol) FBS was added to the cells.

**Analysis of Gene Expression.** The *C. elegans unc-22* gene encodes an abundant striated muscle component that results in a characteristic twitching phenotype. Animals were scored for the twitching phenotype as described (16). GFP expression was assessed in mammalian cells by fluorescence-activated cell sorter (FACS; FacsCaliber, Becton Dickinson) by using pcDNA3.CAT-transfected cells to control for background fluorescence. CAT expression was assessed by using an ELISA-based assay (Roche Molecular Biochemicals). Total protein was determined by using the Bradford method as described (27). Poly(A)<sup>+</sup> RNA was purified from MEFs by using GTC extraction, oligo(dT) cellulose chromatography, and DNase digestion to remove residual plasmid DNA. After electrophoresis [1.2% agarose/1  $\times$  4-morpholinepropanesulfonic acid (Mops)/5.0% formaldehyde] and Northern blot transfer, filters were sequentially hybridized with random prime-labeled cDNA probes corresponding to *egfp* and *neo*. Hybridization intensities were measured by using a BAS150 PhosphorImager (Molecular

**Table 1. Short RNase III-like products can induce specific interference in *C. elegans***

Injection	Fraction affected (number scored)
<i>unc-22</i> siRNA 23 nts	1.4% (145)
<i>unc-22</i> siRNA 24 nts	3.6% (279)
<i>unc-22</i> siRNA 25 nts	16.3% (768)
<i>unc-22</i> sense ssRNA 25 nts	0% (>1100)
<i>unc-22</i> antisense ssRNA 25 nts	0% (>600)
<i>unc-22</i> dsRNA 81 nts	88.9% (180)
<i>egfp</i> siRNA 22 nts	0% (>300)
<i>egfp</i> siRNA 23 nts	0% (>300)
<i>egfp</i> siRNA 24 nts	0% (>300)
<i>egfp</i> siRNA 25 nts	0% (>300)
No injection	0% (>300)

dsRNA molecules were formed with each strand carrying a 5'-PO<sub>4</sub>, 3'-OH, and 2-base 3' overhangs. These were injected into adult *C. elegans* as described in *Methods*. Percentages shown denote portion of progeny broods that show a specific decrease in *unc-22* function as evidenced by twitching behavior in 330  $\mu$ M levamisole. Numbers in parenthesis are total numbers of animals scored. nts, nucleotides.

Dynamics), and pixel densities were calculated by using IMAGE READER 1.4 and IMAGE GAUGE 3.0 (Fuji).

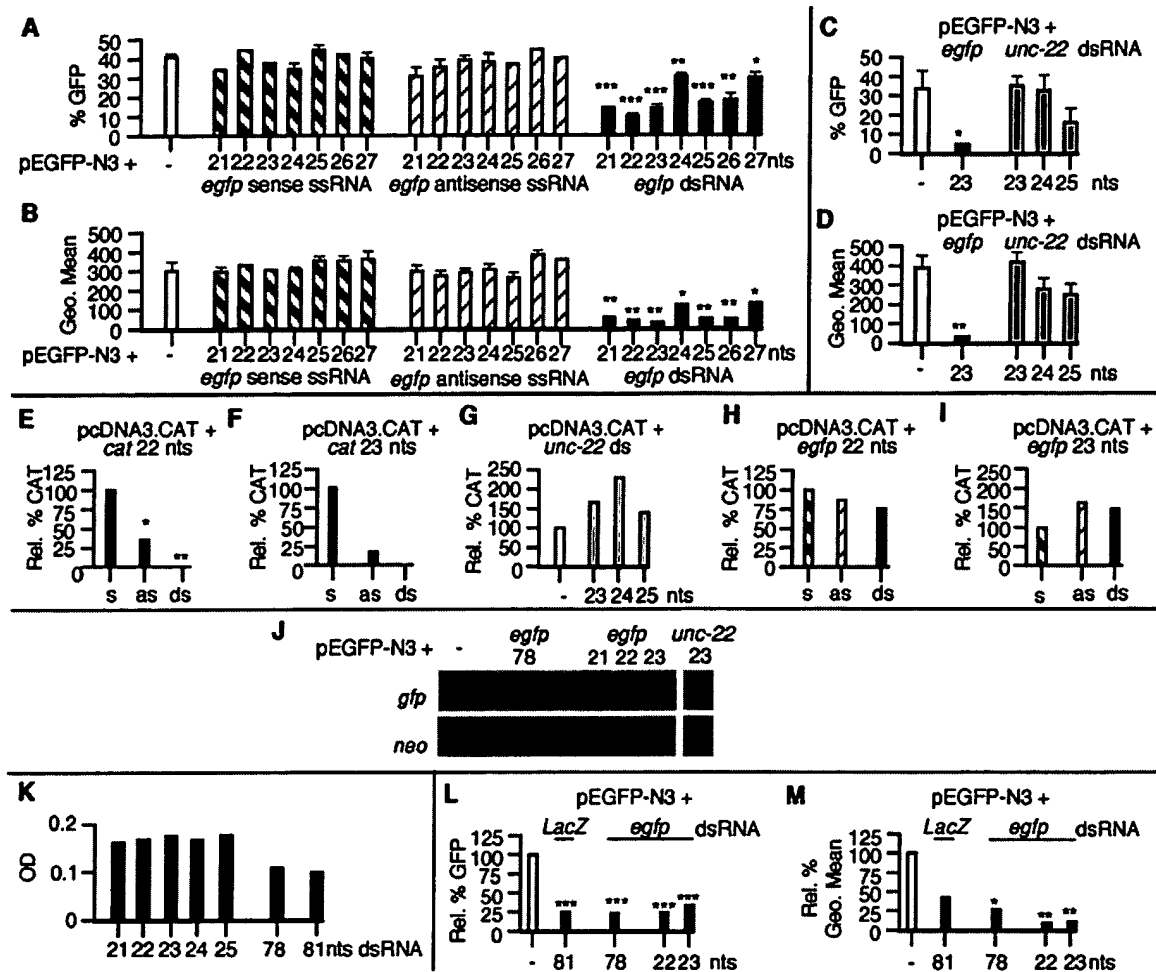
**Cell Survival and *in Vitro* Kinase Assays.** To assay cell survival, MEFs were plated in 96-well plates  $\approx$ 18 h before transfection and were transfected at  $\approx$ 70–80% confluency by using Lipofectamine as a carrier. RNA transfections were conducted as above, except for the omission of the plus complexation step, and using 1/10th the amount of RNA and lipid and 1/10th the volume of medium. Cell viability was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) labeling reagent as described by the manufacturer (Roche Molecular Biochemicals) 48 h after initiation of transfection. *In vitro* kinase reactions were conducted in a final volume of 12.5 ml by using 100 mM [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 1 Ci/mM, Amersham Pharmacia), 100 mM ATP (Sigma) in 20 mM Hepes (pH 7.5), 90 mM KCl, 5 mM MgOAc, 1 mM DTT, and an equal amount of cell lysate prepared from 1  $\times$  10<sup>6</sup> human Jurkat T lymphocytes treated with 100 units/ml of rhIFN- $\beta$  for 24 h before lysis (lysis buffer: 20 mM Hepes/120 mM KCl/5 mM MgOAc/1 mM benzamidine/1 mM DTT/1% Nonidet P-40). dsRNA (1  $\mu$ g/ml) was added to each reaction mixture, and the reactions were incubated for 10 min at 30°C. Reactions were quenched by addition of an equal volume of 2 times sample buffer (2 times sample buffer: 62.5 mM Tris-Cl, pH 6.8/10% glycerol/2% SDS/0.0125% bromophenol blue/5%  $\beta$ -mercaptoethanol), boiled for 2 min, and subjected to electrophoresis [10% (vol/vol) SDS/PAGE]. Labeled proteins were visualized by autoradiography of dried gels.

## Results

**Short RNase III-Like Products Can Induce Inhibition of Gene Expression in Invertebrate Cells.** A series of dsRNAs with characteristics of siRNAs (5' phosphate, 3' hydroxyl, and 2 base 3' overhangs on each strand) were generated from chemically synthesized ssRNAs. The siRNAs varied from 21–27 nucleotides and had sequences that matched three different target RNAs, *unc-22*, *cat*, and *egfp* (for sequences see Table 2).

To determine whether siRNAs can be used directly to inhibit gene expression we first assessed interference in *C. elegans* by using siRNAs corresponding to *C. elegans unc-22* (Table 1). *unc-22* provides a sensitive and specific assay for genetic interference as this is the only gene in the *C. elegans* genome that can mutate by loss of function to give a twitching phenotype. *unc-22*

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**Fig. 1.** Gene-specific inhibition of expression in MEFs by siRNAs. MEFs transfected with plasmid DNA, ssRNAs, and dsRNAs were harvested 48 h after transfection and were assayed for (A–D) GFP expression by FACS analysis (each transfection was assayed in triplicate and data are shown as mean  $\pm$  SEM). A and C show the percentage of GFP-positive cells and B and D show the fluorescence intensity (Geo Mean) of GFP-positive cells. (E–I) CAT expression (each transfection condition was assayed in triplicate; data in E are normalized to the amount of CAT pg/ $\mu$ g of protein observed in pcDNA3.CAT-transfected cells; data in F–I are normalized to the amount of CAT pg/ $\mu$ g of protein in plasmid and sense ssRNA-transfected cells. s, sense ssRNA; as, antisense ssRNA). (J) *egfp* and *neo* RNA levels by Northern analysis of poly(A)<sup>+</sup> mRNA. (K) Cell survival (assayed in duplicate and shown as a mean OD<sub>500–650</sub>; dsRNAs of 21–25 and 78 nucleotides correspond to *egfp*; the dsRNA of 81 nucleotides corresponds to *LacZ*). (L and M) GFP expression by FACS analysis (data are shown as relative percentage normalized to pEGFP-N3-transfected cells). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

siRNAs induced a decrease in *unc-22* gene expression as measured by the presence of the twitching phenotype in the progeny of injected adults. Small dsRNAs of 23, 24, and 25 nucleotides produced interference with the 25-nt *unc-22* siRNA inducing the highest fraction of animals with an affected phenotype (16.3%). As a control, siRNAs directed against an unrelated sequence (*egfp*) induced no phenotypic changes (Table 1).

**21–23-nt dsRNAs Inhibit Expression in MEFs.** To test whether small dsRNA molecules can specifically inhibit gene expression in vertebrate cells, we cotransfected MEFs with expression plasmids encoding GFP (pEGFP-N3) and CAT (pcDNA3.CAT), and synthetic siRNAs corresponding to *egfp*, *cat*, or *unc-22* (Fig. 1). The *egfp* dsRNAs (21–27 nucleotides) all inhibited GFP expression in MEFs. The 22- and 23-nt *egfp* siRNAs (20 and 21 nucleotides base-paired with 2-nt 3' overhangs) showed the greatest degree of inhibition, both with respect to the total number of cells expressing GFP (Fig. 1A) and the fluorescence intensity of the GFP expression observed in GFP-positive cells

(Fig. 1B). In contrast, *unc-22* dsRNAs of 23–25 nucleotides had no significant effect on GFP expression (Fig. 1C and D).

To further assess the efficacy and specificity of the inhibition mediated by siRNAs in mammalian cells, we used a second reporter, CAT (Fig. 1E–I). *cat* siRNAs of 22 and 23 nucleotides completely inhibited CAT expression (Fig. 1E and F), whereas *unc-22* and *egfp* dsRNAs had no little or no effect on CAT expression (Fig. 1G–I). Although no antisense effect had been seen by using GFP as a reporter, the *cat* ssRNA antisense oligomers partially inhibited CAT expression. However, the siRNA-mediated inhibition was more potent ( $\approx 1.5$ -fold), suggesting that the gene silencing mediated by the small dsRNAs can be distinguished from a purely antisense-based mechanism.

To analyze this inhibition of *egfp* expression at an RNA level, poly(A)<sup>+</sup> RNA was purified from transfected MEFs and subjected to Northern analysis by using cDNA probes corresponding to *egfp* and *neo*, both encoded by the pEGFP-N3 plasmid (Fig. 1J). Quantitative PhosphorImager analysis showed a decrease in the levels of the *egfp* mRNA obtained from cells cotransfected

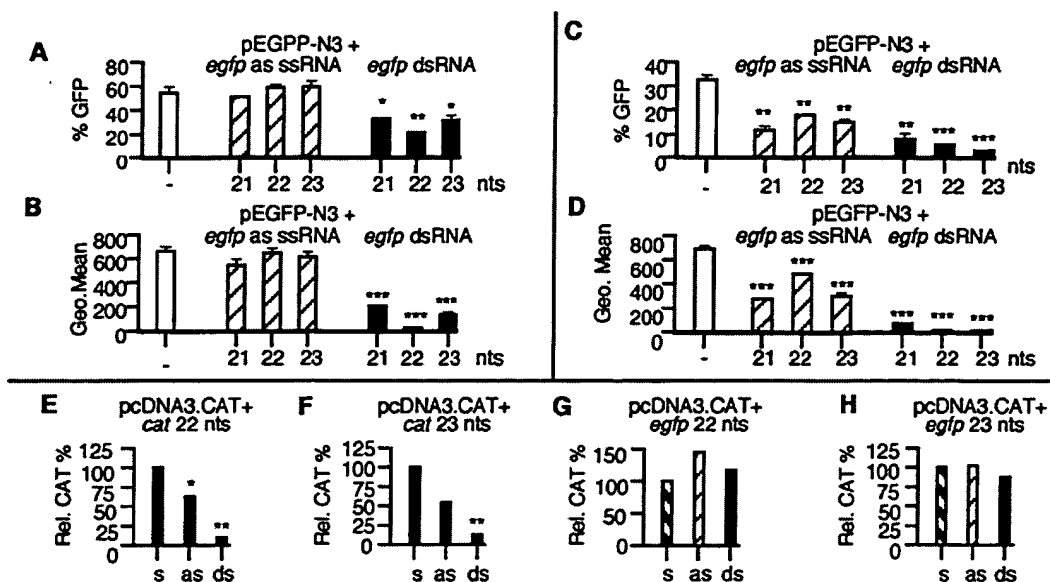


Fig. 2. siRNA-mediated gene silencing in human cells. (A and B) 293 and (C and D) HeLa cells transfected with pEGFP-N3 and antisense (as) ssRNAs and dsRNAs were harvested 48 h after transfection and were assayed for GFP expression by FACS analysis (assayed in triplicate; data are shown as mean  $\pm$  SEM). A and C show the percentage of GFP-positive cells and B and D show the fluorescence intensity (Geo Mean) of GFP-positive cells. (E-H) HeLa cells transfected with pcDNA3-CAT, ssRNAs, and dsRNAs were harvested 48 h after transfection and assayed for CAT expression (assayed in triplicate and normalized to the amount of CAT pg/ $\mu$ g of protein observed in plasmid plus sense-transfected cells. s, sense ssRNA; as, antisense ssRNA). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

with the pEGFP-N3 plasmid and the 21-, 22-, and 23-nt *egfp* siRNAs, compared with cells transfected only with the GFP plasmid. The percentage decrease was  $\approx 60\%$  for all three *egfp* siRNAs when compared with the levels of *egfp* mRNA in cells transfected only with plasmid. Importantly, no effect was seen on the levels of the *neo* transcript compared with plasmid-only transfected cells, indicating that the inhibition induced by the small *egfp* dsRNAs was sequence-specific. Consistent with this hypothesis, the 23-nt dsRNA corresponding to the *C. elegans unc-22* gene had no effect on either *egfp* or *neo* expression.

To follow the fate of cells transfected with siRNAs and larger dsRNAs, we assayed MEF cell survival (Fig. 1K). Longer dsRNAs (78 or 81 nucleotides with flush ends) induced a substantial degree of cell death (up to 50%) in a 48-h period, whereas the smaller dsRNAs had a minimal effect on the growth of cells. By examining the effect of the larger dsRNAs on gene expression, we observed that the larger dsRNAs (78 or 81 nucleotides) induced a sequence nonspecific decrease of 75% in the percentage of cells expressing GFP (Fig. 1L) and in CAT protein levels (data not shown), compared with plasmid controls. This nonspecific decrease in gene expression is consistent with previous data from numerous mammalian systems and contrasts with the specific gene silencing the 78-nt *egfp* dsRNA induces in *Drosophila* S2 cells (27). However, it should be noted that the decrease in transgene expression after siRNA transfection could be distinguished from the nonspecific inhibition by examination of the GFP fluorescence intensity seen in viable cells. The fluorescence intensity of GFP expression best illustrates a change in the total amount of GFP made by a live cell and therefore is less influenced by nonspecific cell death. Although some decrease ( $\approx 60\%$ ) in the fluorescence intensity was seen by using the larger  $\approx 80$ -nt dsRNA molecules (irrespective of sequence), the *egfp* siRNAs of 22 and 23 nucleotides consistently reduced the intensity of the GFP signal (by  $\approx 90\%$ ) to near background levels (Fig. 1M). The difference in specificity between the longer dsRNAs and siRNAs could also be seen at an RNA level where the 78-nt *egfp* dsRNA induced a significant

decrease in both the *egfp* and *neo* transcripts, whereas the siRNAs inhibited only *egfp* (Fig. 1J).

**Inhibition of Gene Expression in Human Somatic Cells.** To date, there has been no evidence of an RNAi-like process occurring in human somatic cells. To determine whether siRNAs could also specifically inhibit gene expression in human cells, we cotransfected two commonly used human cell lines, the embryonic kidney cell line 293 and the epithelial carcinoma cell line HeLa, with plasmids and RNA (Fig. 2). All of the *egfp* siRNAs tested inhibited GFP gene expression in 293 (Fig. 2A and B) and HeLa (Fig. 2C and D) cells, with the 22- and 23-nt *egfp* siRNAs inducing the greatest decrease in GFP expression. In 293 cells cotransfected with pEGFP-N3 and the 22-nt *egfp* siRNA, the intensity of GFP expression was reduced to near background levels (Fig. 2B). Similar results were seen in HeLa cells cotransfected with pEGFP-N3 and the 22- or 23-nt *egfp* siRNAs (Fig. 2D). dsRNAs corresponding to *unc-22* had no effect on GFP expression in these cells (data not shown). The siRNA-triggered inhibition of GFP expression was dose-dependent in that doubling the amount of dsRNA (from 70 to 140 pmols) decreased GFP intensity by an additional 25% for the *egfp* 22-nt siRNA and by 45% for the *egfp* 23-nt siRNA. CAT expression was also significantly inhibited by siRNAs corresponding to *cat* (Fig. 2E and F) in HeLa cells. Again, the inhibition mediated by the siRNAs was significantly higher than that seen by using ssRNA antisense oligomers. Cotransfection of the pcDNA3.CAT plasmid and the *egfp* siRNAs of the same size and of similar GC/AT complexity had no effect on CAT expression (Fig. 2G and H).

**siRNA-Mediated Inhibition of Gene Expression Is Independent of Nonspecific Interference Pathways Activated by Larger dsRNAs.** It has been reported that small blunt-ended dsRNAs of less than 30 bp do not activate PKR (34). Indeed, at high concentrations these short dsRNAs can competitively inhibit activation of PKR by larger dsRNAs. Similarly, the synthetic siRNAs used in this study did not activate PKR (Fig. 3A) and inhibited the activation of

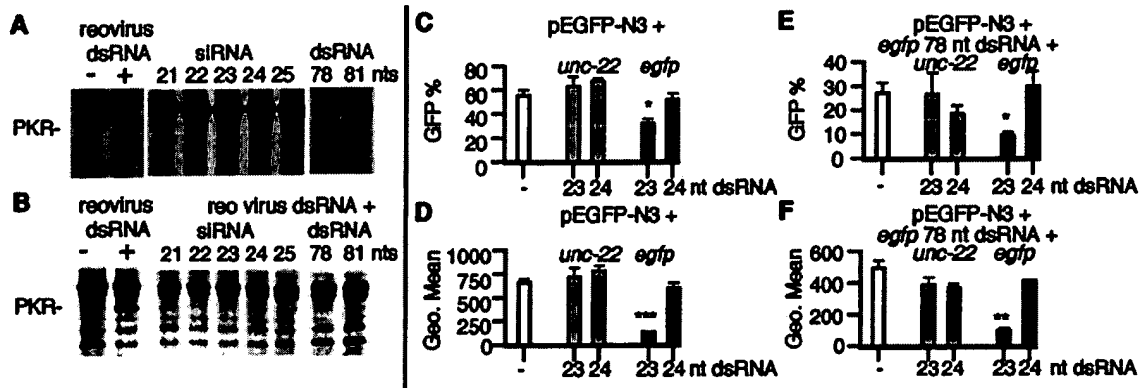


Fig. 3. siRNAs and mammalian dsRNA-dependent pathways. To detect PKR autophosphorylation, we performed *in vitro* kinase assays as described in Methods. (A) *In vitro* kinase reactions were performed without exogenous RNA (–) or with 1  $\mu$ g/ml of reovirus dsRNA or 1  $\mu$ g/ml of siRNA (21–25 nucleotides), or 1  $\mu$ g/ml of 78- or 81-nt dsRNA. (B) *In vitro* kinase competition assays were performed by using si- and dsRNAs. Reactions were performed without exogenous RNA (–) or 1  $\mu$ g/ml of reovirus RNA, or 75-fold excess siRNA (21–25 nucleotides) or 78- or 81-nt dsRNA, plus reovirus dsRNA (1  $\mu$ g/ml). siRNAs of 21–25 nucleotides and dsRNA of 78 nucleotides corresponded to *egfp* (the 81-nt dsRNA corresponds to *LacZ*). (C and D) 293 cells transfected with pEGFP-N3 and *unc-22* or *egfp* siRNAs, and (E and F) 293 cells transfected with pEGFP-N3 and 78 *egfp* dsRNA and *unc-22* or *egfp* siRNAs were assayed for GFP expression by FACS analysis 48 h after transfection (each transfection was assayed in triplicate; data are shown as mean  $\pm$  SEM). B and D show the percentage of GFP-positive cells and C and E show the fluorescence intensity (Geo Mean) of GFP-positive cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

PKR by a large viral dsRNA (Fig. 3B). Interestingly, in this assay we were unable to detect activation of PKR by the 78- and 81-nt dsRNAs, despite observing a substantial level of cell death, suggesting that other dsRNA-dependent kinases or other pathways may be contributing in MEFs to the decrease in gene expression and cell death observed with these RNAs.

To see whether the small dsRNAs could block the toxic effect of the larger dsRNAs in cells, we cotransfected 293 cells with the pEGFP-N3 plasmid, the *egfp* 78-nt dsRNA, and the *unc-22* 23- or 24-nt siRNAs or the *egfp* 23- or 24-nt siRNAs (Fig. 3 C–F). The cell death induced by the 78-nt *egfp* dsRNA was not inhibited by the *unc-22* or *egfp* siRNAs (Fig. 3 C and D vs. E and F) but importantly the 78-nt *egfp* dsRNA did not block the specific inhibition of GFP expression mediated by the 23-nt *egfp* siRNA. This result suggests that the siRNA-mediated gene silencing mechanism is independent of nonspecific responses of mammalian cells to dsRNA.

## Discussion

A consistent observation of PTGS and RNAi in several species has been the detection of small dsRNAs ( $\approx 21$ –25 nucleotides) and siRNAs derived from the triggering dsRNA. These small dsRNAs have been observed irrespective of whether the initiating dsRNA is delivered directly, is derived from a viral RNA, or is produced from a transgene (12–17). These findings and further biochemical analysis (18) have suggested that the generation of siRNAs represents a critical step in the RNAi/PTGS mechanism. We now present evidence that these siRNAs can have direct effects on gene expression in *C. elegans* and mammalian cell culture *in vivo*. Our results in mammalian cells are particularly striking in that previous attempts to assay RNAi effects in vertebrate somatic cells have encountered effects that were predominantly gene-nonspecific (26–29). We propose that the small size of the siRNAs avoids the induction of the nonspecific responses of mammalian cells to dsRNA.

Several models have been put forward to explain RNAi, in particular the mechanisms by which siRNAs interact with the target mRNA and thus facilitate its degradation (12–15, 17, 37). It has been proposed that the siRNAs act as a guide for the enzymatic complex required for the sequence-specific cleavage of the target mRNA. Evidence for the role of siRNA as a guide includes cleavage of the target mRNA at regular intervals of

$\approx 21$ –23 nucleotides in the region corresponding to the input dsRNA (13), with the exact cleavage sites corresponding to the middles of sequences covered by individual 21- or 22-nt siRNAs (17). Although mammals and lower organisms seem to share dsRNA-triggered responses that involve a related intermediate (siRNAs), it is likely that there will be differences as well as similarities in the underlying mechanism.

Several of the proteins shown to play key roles in RNA-triggered gene silencing in plants and invertebrates share homology with potential coding regions from the human or other vertebrate genomes. These include putative RNA-dependent polymerases (RdRp; refs. 38–41), the RDE-1/Argonaute family (8), and a variety of putative helicases and nucleases (9, 18, 42–44). Mammalian homologs of the RNAi-associated *Drosophila* RNase III have been identified (45, 46). Importantly, one of these putative RNases has been shown to generate small dsRNA molecules of  $\approx 22$  nucleotides from larger dsRNAs (18). However, even in invertebrate systems, the precise role of these factors in RNAi remains to be elucidated. Because factors from each of these homology classes have identified roles in normal physiology and development (i.e., beyond genome surveillance), a full analysis of the reaction mechanisms in the different biological systems may be needed before a clear picture of the commonality between RNAi in these different systems will emerge.

Our experiments do not address possible differences in mechanism between invertebrate and vertebrate systems, although we observed some variation between the different assay systems in the optimal size and effectiveness of the inhibiting dsRNA. These differences could be gene-, species-, cell type-, or assay-specific; it will be particularly interesting to determine whether there are species-dependent differences in the length or structure of natural siRNAs. It is not yet clear what roles RNAi/PTGS might play in mammalian systems. RNAi-related silencing mechanisms in plant and invertebrate systems have been implicated in the silencing of viruses and transposons. Mammalian genomes have a need to cope with a considerable load of viruses, selfish DNA, and aberrant transcription. RNAi-related mechanisms could certainly function as a part of the defense network for any or all of these genomic hazards. Alternatively, specific gene silencing by dsRNA could function in normal mammalian gene regulation, e.g., in imprinting or X inactivation (47).

Because of the efficacy and ease with which RNAi can be induced, RNAi has been rapidly exploited in *C. elegans* and *Drosophila* as a reverse genetics tool (48). Currently, the principal method used to reduce gene expression in mammalian cells utilizes antisense sequences in the form of single-stranded oligonucleotides and transcripts. The interaction of antisense sequences with mRNA through Watson-Crick base-pairing leads to a decrease in gene expression by several possible mechanisms, including the activation of RNaseH, which cleaves RNA/DNA duplexes, and the inhibition of RNA processing and/or translational blockade (49). Several issues have limited wider use of antisense technology. Problems have included a lack of suitable target sequences within a given mRNA caused by RNA secondary folding, which necessitates screening of multiple antisense sequences to identify those that mediate the greatest level of inhibition and inefficient delivery *in vitro* and *in vivo*. We have tested only a limited number of siRNAs in mammalian cells but as yet all of the siRNAs that were tested produced specific

inhibition of gene expression, and the siRNAs seem to be very stable and thus may not require the extensive chemical modifications that ssRNA antisense oligonucleotides require to enhance the *in vivo* half-life. Our initial experiments suggest that siRNAs may be useful for triggering RNAi-like responses that could be used as functional genomics and therapeutic tools. Certain applications may be facilitated by the simple transfection protocols that we have used, whereas other applications may benefit from further optimization and additional exploration of the RNAi mechanism.

**Note.** A recent report by Elbashir *et al.* (50) describes a specific interference response in mammalian cells by using 21-nt siRNAs.

We thank Phil Sharp, Lisa Timmons, Patrick Weiss, Mario Mautino, and members of the Fire Laboratory for insightful discussions, and John Bell for MEFs. We thank the National Institutes of Health (AF R01-GM37706, T32-GM07321, and FI A1446962) and the Carnegie Institution for support. N.J.C. is a National Institutes of Health Fogarty Fellow.

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Supplementary material for Caplen *et al.* (July 31, 2001) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.171251798.

**Table 2. Sequences of RNA oligonucleotides**

RNA oligonucleotide	Sequence
<i>egfp</i> 21-nt sense	5' PO <sub>4</sub> r(gac gua aac ggc cac aag uuc) 3' OH (nt 64–nt 84)
<i>egfp</i> 21-nt antisense	5' PO <sub>4</sub> r(acu ugu ggc cgu uua cgu cgc) 3' OH (nt 62–nt 82)
<i>egfp</i> 22-nt sense	5' PO <sub>4</sub> r(gca agc uga ccc uga agu uca u) 3' OH (nt 122–nt 143)
<i>egfp</i> 22-nt antisense	5' PO <sub>4</sub> r(gaa cuu cag ggu cag cuu gcc g) 3' OH (nt 120–nt 141)
<i>egfp</i> 23-nt sense	5' PO <sub>4</sub> r(agg agc acg acu ucu uca agu cc) 3' OH (nt 239–nt 261)
<i>egfp</i> 23-nt antisense	5' PO <sub>4</sub> r(acu uga aga agu cgu gcu gcu uc) 3' OH (nt 237–nt 259)
<i>egfp</i> 24-nt sense	5' PO <sub>4</sub> r(cau cuu cuu caa gga cga cgg caa) 3' OH (nt 294–nt 317)
<i>egfp</i> 24-nt antisense	5' PO <sub>4</sub> r(gcc guc guc cuu gaa gaa gau ggu) 3' OH (nt 292–nt 315)
<i>egfp</i> 25-nt sense	5' PO <sub>4</sub> r(gca caa gcu gga gua caa cua caa c) 3' OH (nt 417–nt 441)
<i>egfp</i> 25-nt antisense	5' PO <sub>4</sub> r(ugu agu ugu acu cca gcu ucu gcc c) 3' OH (nt 415–nt 439)
<i>egfp</i> 26-nt sense	5' PO <sub>4</sub> r(cga caa gca gaa gaa cgg cau caa gg) 3' OH (nt 465–nt 490)
<i>egfp</i> 26-nt antisense	5' PO <sub>4</sub> r(uug aug ccg uuc uuc ugc uug ucg gc) 3' OH (nt 463–nt 488)
<i>egfp</i> 27-nt sense	5' PO <sub>4</sub> r(cuu caa gau ccg cca caa cau cga gga) 3' OH (nt 495–nt 521)
<i>egfp</i> 27-nt antisense	5' PO <sub>4</sub> r(cuc gau guu gug gcg gau cuu gaa guu) 3' OH (nt 493–nt 519)
<i>cat</i> 22-nt sense	5' PO <sub>4</sub> r(gag uga aua cca cga cga uuu c) 3' OH (nt 318–nt 339)
<i>cat</i> 22-nt antisense	5' PO <sub>4</sub> r(aau cgu cgu ggu auu cac ucc a) 3' OH (nt 316–nt 337)
<i>cat</i> 23-nt sense	5' PO <sub>4</sub> r(gga gug aaU acc acg acg auu uc) 3' OH (nt 317–nt 339)
<i>cat</i> 23-nt antisense	5' PO <sub>4</sub> r(gga gug aaU acc acg acg auu uc) 3' OH (nt 315–nt 337)
<i>unc22</i> 23-nt sense	5' PO <sub>4</sub> r(ucu guc ucu gcu ccu cuc ggc gg) 3' OH (nt 17893–nt 17915)
<i>unc22</i> 23-nt antisense	5' PO <sub>4</sub> r(gcc gag agg agc aga gac aga gg) 3' OH (nt 17891–nt 17913)
<i>unc22</i> 24-nt sense	5' PO <sub>4</sub> r(cuc ugu cuc ugc ucc ucu cgg cgg) 3' OH (nt 17892–nt 17915)
<i>unc22</i> 24-nt antisense	5' PO <sub>4</sub> r(gcc gag agg agc aga gac aga gga) 3' OH (nt 17890–nt 17913)
<i>unc22</i> 25-nt sense	5' PO <sub>4</sub> r(ccu cug ucu cug cuc cuc ucg gcg g) 3' OH (nt 17891–nt 17915)
<i>unc22</i> 25nt antisense	5' PO <sub>4</sub> r(gcc gag agg agc aga gac aga gga u) 3' OH (nt 17889–nt 17913)
<i>egfp</i> 78-nt sense	5' r(cua cgu cca gga gcg cac cau cuu cuu caa gga cga cgg caa cua caa gac ccg cgc cga ggu gaa guu cga ggg cga) 3' (nt 276–nt 354)
<i>egfp</i> 78-nt antisense	5' r(ucg ccc ucg aac uuc acc ucg gcg cgg guc uug uag uug ccg ucg ucc uug aag aag aug gug cgc ucc ugg acg uag) 3' (nt 276–nt 354)
<i>cat</i> 78-nt sense	5' r(cau cgc ucu gga gug aaU acc acg acg auu ucc ggc agu uuc uac aca uau auu cgc aag aug ugg cgu guu acg gug) 3' (nt 313 – nt 392)
<i>cat</i> 78-nt antisense	5' r(cac cgu aac acg cca cau cuu gcg aaU aua ugu gua gaa acu gcc gga aaU cgu cgu ggu auu cac ucc aga gcg aug) 3' (nt 313–nt 392)
<i>unc</i> 22 81-nt sense	5' r(gug cuc gga aaa cca ucu agc cca uug gga ccu uug gaa gug ucg aaU guc uac gaa gau cgc gca gau uug gag ugg aaa) 3' (nt 4096–nt 4176)
<i>unc</i> 22 85-nt antisense	5' r(gua cuu ucc acu cca aaU cug cgc gau cuu cgu aga cau ucg aca cuu cca aag guc cca aug ggc uag aug guu uuc cga gca c) 3' (nt 4096–4181 nt)
<i>LacZ</i> 81-nt sense	5' r(agg ucc ugc acu gga ugg ugg cgc ugg aug gua agc cgc ugg caa gcg gug aag ugc cuc ugg aug ugc cuc cac aag gua) 3' (nt 1946–nt 2027)
<i>LacZ</i> 81-nt antisense	5' r(uac cuu gug gag cga cau cca gag gca cuu cac cgc uug cca gcg gcu uac cau cca gcg cca cca ucc agu gca gga gcu) 3' (nt 1946–nt 2027)

Sequence positions refer to the 719-nt coding region of *egfp* gene, the 675-nt coding region of the *cat* gene, the 3,066-nt coding region of the  $\beta$ -galactosidase gene, and the 18,147-nt coding of the *C. elegans unc22* gene.

# RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase

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Received February 28, 2002; Revised and Accepted March 25, 2002

## ABSTRACT

Methods that allow the specific silencing of a desired gene are invaluable tools for research. One of these is based on RNA interference (RNAi), a process by which double-stranded RNA (dsRNA) specifically suppresses the expression of a target mRNA. Recently, it has been reported that RNAi also works in mammalian cells if small interfering RNAs (siRNAs) are used to avoid activation of the interferon system by long dsRNA. Thus, RNAi could become a major tool for reverse genetics in mammalian systems. However, the high cost and the limited availability of the short synthetic RNAs and the lack of certainty that a designed siRNA will work present major drawbacks of the siRNA technology. Here we present an alternative method to obtain cheap and large amounts of siRNAs using T7 RNA polymerase. With multiple transfection procedures, including calcium phosphate co-precipitation, we demonstrate silencing of both exogenous and endogenous genes.

## INTRODUCTION

Over the last few years, RNA interference (RNAi) has been recognized as a major mechanism of post-transcriptional gene silencing in the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila*, as well as in plants (1). This phenomenon is based on double-stranded RNA (dsRNA) that triggers the silencing of gene expression in a sequence-specific manner. According to the prevailing model, the injected or transfected dsRNA is processed into small RNAs (guide RNAs or small interfering RNAs, siRNAs) of 21–25 nt, depending on the species (2,3). The siRNAs probably associate with a multi-component nuclease, identified in *Drosophila* and called RISC (RNA-induced silencing complex), and guide this enzyme for sequence-specific degradation of the mRNA (4). In mammalian cells, the interferon-mediated antiviral response to long dsRNA that leads to the shutdown of protein synthesis precludes the use of RNAi (5). To bypass this non-specific effect, short interfering dsRNAs of 21 nt (which do not activate the antiviral response) have been used instead (6). The function of several endogenous genes has recently been investigated with this technique in mammalian cells (7,8). This new

approach requires the chemical synthesis of short RNAs involving high cost without a guarantee that the purchased siRNA will be effective in silencing (6). To alleviate these problems, we present a simple alternative to obtain large amounts of short interfering RNAs with T7 RNA polymerase-directed *in vitro* transcription. The obtained siRNAs promote silencing in different mammalian cells of both exogenous and endogenous genes.

## MATERIALS AND METHODS

### T7 siRNA synthesis

Desalted DNA oligonucleotides were ordered from Microsynth (Switzerland). (i) T7, 5'-TAATACGACTCACTA-TAG-3'. (ii) GFP as in Caplen *et al.* (9): sense, 5'-ATGAACITTCAGGGTCAGCTTGCTATAGTGAGTCGTATTA-3'; antisense, 5'-CGGCAAGCTGACCCTGAAGTTCTA-TAGTGAGTCGTATTA-3'. (iii) PKR nucleotides 931–949 relative to the start codon: sense, 5'-AAGATCAAGTTTT-GCCAATGCTATAGTGAGTCGTATTA-3'; antisense, 5'-AAGCATTGGCAAACTTGATCTATAGTGAGTCGTATTA-3'. The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase has been described (10). For each transcription reaction, 1 nmol of each oligonucleotide was annealed in 50 µl of TE buffer (10 mM Tris-HCl pH 8.0, and 1 mM EDTA) by heating at 95°C; after 2 min, the heating block was switched off and allowed to cool down slowly to obtain dsDNA. Transcription was performed in 50 µl of transcription mix: 1× T7 transcription buffer (40 mM Tris-HCl pH 7.9, 6 mM MgCl<sub>2</sub>, 10 mM DTT, 10 mM NaCl and 2 mM spermidine) 1 mM rNTPs, 0.1 U yeast pyrophosphatase (Sigma), 40 U RnaseOUT (Life Technologies) and 100 U T7 RNA polymerase (Fermentas) containing 200 pmol of the dsDNA as template. After incubation at 37°C for 2 h, 1 U RNase free-DNase (Promega) was added at 37°C for 15 min. Sense and antisense 21-nt RNAs generated in separate reactions were annealed by mixing both crude transcription reactions, heating at 95°C for 5 min followed by 1 h at 37°C to obtain 'T7 RNA polymerase synthesized small interfering double-stranded RNA' (T7 siRNA). The mixture (100 µl) was then adjusted to 0.2 M sodium acetate pH 5.2, and precipitated with 2.5 vol ethanol. After centrifugation, the pellet was washed once with 70% ethanol, dried, and resuspended in 50 µl of water.

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## Cell culture

Human HeLa and HEK293T cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 5% fetal calf serum, glutamine, penicillin and streptomycin. Cells were seeded into 6-well plates in 2.5 ml of medium 2–3 h prior to transfection by the calcium phosphate co-precipitation technique. Unless noted, 2 µl of T7 siRNA plus 1 µg/well of plasmids pEGFP-C1 (Clontech) and pcDNA3-Luc (11) in 90 µl of water were prepared in a tube to which 30 µl of 1 M CaCl<sub>2</sub> was added. The calcium/DNA/siRNA solution was mixed quickly with 120 µl of 2× phosphate solution (140 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM HEPES, pH 7.02) and the precipitate was immediately added to the wells. For silencing of an endogenous gene, 2 µl of T7 siRNA plus 2 µg of vector pcDNA3 were transfected by the calcium phosphate co-precipitation technique. For liposome-mediated transfection, cells were seeded into 6-well plates 4–5 h prior to treatment. Co-transfection of plasmids and T7 siRNAs was carried out with Lipofectamine (Life Technologies) as directed by the manufacturer for adherent cell lines. Unless noted otherwise, 2 µl of T7 siRNA and 0.5 µg of pEGFP-C1 formulated into liposomes were applied per well. The final volume was 1 ml/well. Cells were harvested and lysed 24 and 40 h later for GFP and PKR analysis, respectively.

## Western blotting

Cells were lysed in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 5% glycerol, protease inhibitors and 1 mM DTT. Equal amounts of total proteins were separated on a 10% polyacrylamide gel and transferred to nitrocellulose. The proteins were revealed either with anti-GFP (Clontech), anti-PKR (Santa-Cruz), anti-luciferase (12) or anti-Hsp90 (a kind gift from Dr David Toft, Mayo Clinic) antibodies.

## RESULTS AND DISCUSSION

To generate siRNAs *in vitro*, we designed the strategy presented in Figure 1. An 18mer oligonucleotide encompassing the T7 promoter is annealed to a 38mer (39mer) oligonucleotide with the complementary sequence of the T7 promoter downstream of the target sequence preceded by two additional nucleotides (reading the sequence 5'→3'). The transcribed sequence is 19 nt (20 nt) plus 2 nt, which can be any nucleotides in the case of the sense RNA but must be complementary nucleotides in the antisense RNA, since it has been shown that the antisense RNA of the siRNA guides target recognition (13). It is noteworthy that T7 RNA polymerase can transcribe a template where only the promoter is double stranded (10). The last guanosine of the T7 promoter is the first ribonucleotide that is incorporated into the RNA by the T7 RNA polymerase during transcription and, therefore, all siRNAs designed by this method will start with a G. Thus, the design of T7 siRNA requires that the sequence starts with a G and has a C at position 19 (position 20) to allow annealing with the complementary RNA, which also starts with a G (see Fig. 1). This G-N17 (N18)-C rule does not restrict the T7 siRNA design since this sequence is frequently found in any gene (on average about five times in a random sequence of 100 bp). To obtain dsRNA for RNAi, only two DNA oligonucleotides

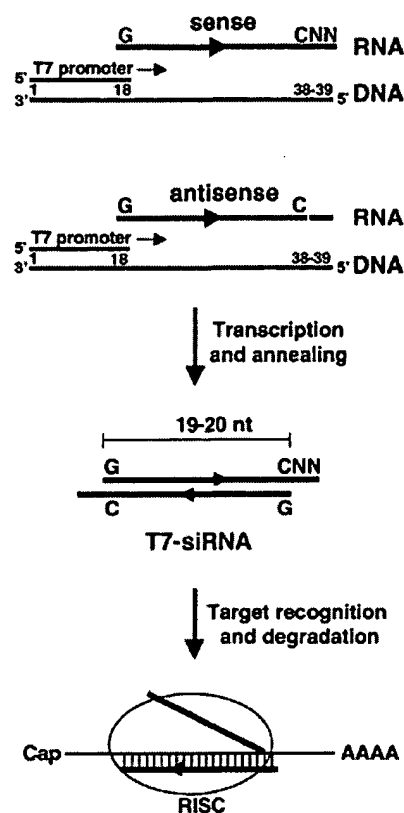
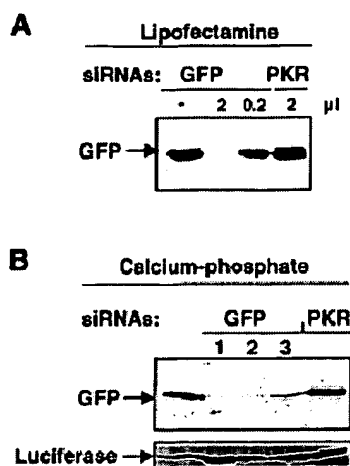


Figure 1. Strategy to generate T7 siRNAs (see Results and Discussion for details). The sequence of the gene of interest is shown in red (sense) or blue (antisense), while the two unrelated nucleotides are in black. RISC stands for the RNA-induced silencing complex that targets the mRNA for cleavage.

corresponding to the sense and antisense sequences of the target gene have to be ordered. The T7 promoter oligonucleotide is invariant and common to any target gene. Following transcription reactions, sense and antisense transcripts are annealed and ethanol precipitated, yielding what we refer to as T7 siRNAs. The integrity of the transcripts was checked on a Nusieve agarose gel (data not shown).

Since our goal was to perform siRNA-mediated silencing with T7 siRNAs, we first selected as a target GFP, which has already been used to study RNAi. The plasmid pEGFP-C1 was transfected together with a 22 nt T7 siRNA into HeLa cells with lipofectamine. Cells were harvested 24 h later and the levels of the exogenously expressed protein were monitored by immunoblot analysis. As shown in Figure 2A, GFP protein levels dropped in the presence of T7 siRNAi targeted to GFP. An unrelated T7 siRNA targeted against the kinase PKR (see below) did not affect GFP levels. Numerous different approaches have been developed to facilitate the transfer of foreign genes into cells, among them the calcium phosphate co-precipitation technique. We thus decided to compare lipofectamine with calcium phosphate. As shown in Figure 2B, GFP expression was also reduced following transfection of T7 siRNAs by the calcium phosphate co-precipitation technique. In the same experiment, the expression of firefly luciferase was not affected by any of the T7 siRNAs, indicating that silencing

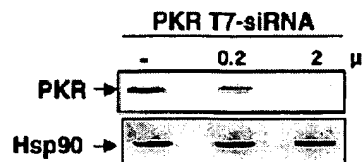


**Figure 2.** T7 siRNAs silence transfected genes in mammalian cells. (A) Plasmid pEGFP-C1 and decreasing amounts of siRNAs were co-transfected using a lipofectamine-based protocol. GFP was revealed with an anti-GFP antibody. T7 siRNA against PKR was transfected as a specificity control. (B) T7 siRNAs directed against GFP (1–3 represent siRNAs from three independent transcription reactions) or PKR were transfected using the calcium phosphate co-precipitation technique into HeLa cells with the plasmids pEGFP-C1 and pDNA3-Luc. GFP and luciferase were detected with anti-GFP and anti-luciferase polyclonal antibodies, respectively.

triggered by T7 siRNAs in mammalian cells is specific, as reported previously for chemically synthesized siRNAs (6,9). Caplen and colleagues used siRNAs of different lengths and showed that both 21- and 22-nt siRNAs work well to silence GFP (9). We have noticed that 22-nt-long T7 siRNAs may be more efficient for silencing transfected genes than 21 nt T7 siRNAs (data not shown). We conclude that T7 siRNAs can be introduced into cells and mediate their silencing effects with the non-liposome based calcium phosphate co-precipitation method.

To complete our study, we tested T7 siRNA on an endogenous gene, the gene for the interferon-induced protein kinase PKR (14). For this target, the T7 siRNA was 21 nt long, as described recently for other endogenous targets (6,7). In this case, the main challenge for a successful RNAi experiment is to achieve a high transfection efficiency. Using either lipofectamine or calcium phosphate co-precipitation, the transfection efficiency reached almost 80% for HeLa and 293T cells (as judged by transfection of GFP; data not shown). As presented in Figure 3, T7 siRNA targeted against human PKR, transfected into 293T by the calcium phosphate co-precipitation technique, was able to down-regulate endogenous PKR 48 h after the beginning of transfection. The silencing was almost completely effective with 2 µl of T7 siRNA. The residual PKR protein observed by immunoblot may reflect the 10–20% untransfected cells. The T7 siRNA was specific since no effect was observed on the unrelated protein Hsp90 (Fig. 3).

In conclusion, we report here a new protocol for the synthesis of siRNAs by T7 RNA polymerase and their transfer into cells. The main advantage of this technique is its simplicity and its extremely low cost compared with the current prices for synthetic RNA oligonucleotides. The amount of T7 siRNA required for each sample is small and represents ~1–5%



**Figure 3.** T7 siRNA silences an endogenous gene in mammalian cells. Aliquots of 0.2 or 2 µl of T7 siRNA directed against PKR were transfected using the calcium phosphate co-precipitation technique into 293T cells. Forty hours after transfection, cells were harvested and equal amounts of proteins were loaded on a 10% polyacrylamide gel. PKR was detected using a polyclonal antibody against PKR. Hsp90 was revealed as a specificity and loading control.

of the synthesized short RNA. We estimate that the average yield of T7 siRNA per transcription reaction is 1–5 µg (per 200 pmol template) and that silencing experiments can be carried out with 40–200 ng of T7 siRNA (in 2 µl; see Figs 2 and 3). But even 10 times less was sufficient for a noticeable decrease in GFP expression (Fig. 2A, lane 0.2). The demonstration that RNAi also works with the calcium phosphate technique further contributes to making this powerful technology widely available and applicable. With our approach, RNA interference in mammalian cells may become as easy as in *C.elegans* or *Drosophila*, where long dsRNAs synthesized by T7 RNA polymerase can be used.

## ACKNOWLEDGEMENTS

We thank David Toft for the Hsp90 antibody. We are grateful to Katharina Strub and Laurent Huck for experimental advice. This work was supported by the Swiss National Science Foundation, Krebs Forschung Schweiz and the Canton de Genève.

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